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Chapter 5

Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation: clinical and genetic characterization and target for therapy

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Brain (accepted for publication)

Abstract

Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation is a disorder caused by recessive mutations in the gene *DARS2*, which encodes mitochondrial aspartyl-tRNA synthetase. Recent observations indicate that the phenotypic range of the disease is much broader than initially thought. Currently, no treatment is available. The aims of our study were (1) to explore a possible genotype-phenotype correlation, and (2) to identify potential therapeutic agents that modulate the splice site mutations in intron 2 of *DARS2*, present in almost all patients.

A cross-sectional observational study was performed in 78 patients with two *DARS2* mutations in the Amsterdam and Helsinki databases up to December 2012. Clinical information was collected via questionnaires and an inventory was made of the *DARS2* mutations in these patients and combined with those previously published. An assay was developed to assess mitochondrial aspartyl-tRNA synthetase enzyme activity in cells. Using a fluorescence reporter system we screened for drugs that modulate *DARS2* splicing.

Clinical information of 66 patients was obtained. The clinical severity varied from neonatal onset, rapidly fatal disease to adult onset disorder with a slow and mild course. The most common phenotype was characterized by childhood onset and slow neurological deterioration. Full wheelchair dependency was rare and usually began in adulthood.

In total, 60 different *DARS2* mutations were identified, 13 of which have not been reported before. Except for four of the 42 cases published by others, all patients were compound heterozygous; 94% of the patients had a splice site mutation in intron 2. The groups of patients sharing the same two mutations were too small for formal assessment of genotype - phenotype correlation. However, some combinations of mutations were consistently associated with a benign phenotype. The mitochondrial aspartyl-tRNA synthetase activity was strongly reduced in patient cells. Among the compounds screened, cantharidin was identified as the most potent modulator of *DARS2* splicing.

In conclusion, the phenotypic spectrum of leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation is wide, but most often the disease has a relatively slow and mild course. The available evidence suggests that the genotype influences the phenotype, but because of the high number of private mutations, larger numbers of patients are necessary to confirm this. The activity of mitochondrial aspartyl-tRNA synthetase is significantly reduced in patients. A compound screen established a 'proof of principle' that the splice site mutation can be influenced. This finding is promising for future therapeutic strategies.

Introduction

Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (LBSL, MIM611105) is a rare autosomal recessive disease that was initially described as a relatively mild disorder, characterized by juvenile onset of slowly progressive ataxia, spasticity and dorsal column dysfunction^{3,4,7-9}. LBSL is associated with a highly distinctive MRI pattern, consisting of signal abnormalities in the periventricular cerebral white matter and specific brain stem and

spinal cord tracts^{3,6}. In most patients, proton magnetic resonance spectroscopy of the abnormal white matter reveals increased lactate^{3,10,11}. The definitive diagnosis of LBSL is established by the demonstration of mutations in the gene *DARS2* (MIM 610956)⁶. No treatment is available for LBSL.

DARS2 encodes mitochondrial aspartyl-tRNA synthetase, the enzyme that attaches the amino acid aspartate to the correct mitochondrial tRNA. Aspartyl-tRNA is necessary in the translation of mitochondrial mRNA into protein. It is striking that almost all patients are compound-heterozygous for two *DARS2* mutations and that one of the mutations is almost invariably a splice site mutation in intron 2, upstream of exon 3⁶. As a consequence of such a mutation, exon 3 is not included in the mRNA, leading to a frameshift, premature stop and absence of functional protein. These splice site mutations are, however, 'leaky'. This means that for part of the mutated mRNAs, exon 3 is included, from which normal full-length protein is formed⁵⁹.

Recent observations indicate that the phenotypic spectrum in LBSL is much wider than originally assumed. Adult-onset oligosymptomatic cases were described^{10,11,58,80}, as well as patients with infantile onset, rapid neurological deterioration and early demise^{57,81,82}. The explanation for this wide clinical variation is unclear.

The first aim of the present study was to explore a possible genotype-phenotype correlation in LBSL. We made an inventory of the clinical characteristics and *DARS2* mutations in a cohort of 78 LBSL patients and evaluated a possible genotype-phenotype relationship. The second aim of the study was to investigate the potential application of agents modulating the common intron 2 splice site mutations, with the aim to increase the amount of normal enzyme produced. A compound screen was performed to identify modulators of the splicing event.

Patients and methods

The study was performed with approval of the Institutional Review Board of the VU University Medical Center, Amsterdam. Written informed consent for research on patients' cells was obtained from all patients, or guardians of the patients, participating in the study, in agreement with the Declaration of Helsinki.

Phenotypic inventory

A cross-sectional observational study was performed including all 68 patients with two *DARS2* mutations present in the Amsterdam LBSL patient database up to December 2012. The database contains patients referred to the VUmc Center for Childhood White Matter Disorders in Amsterdam for genetic testing. Additionally, 10 Finnish patients, investigated in the Helsinki University Central Hospital, were included.

Clinical questionnaires were completed primarily by the physician following the patient (77% of the patients). If this source was not available, the information was derived from medical records, supplemented by information provided by the family. In order to avoid differences in rating of the clinical phenotype, robust outcome measures were chosen: age of onset, age at achieving unsupported walking, age at loss of walking without support, full wheelchair dependency, gross

motor function classification system (GMFCS, Expanded and Revised version) and manual ability classification system (MACS) scores, cognitive ability and age at death. The GMFCS and the MACS are international standards for the classification of locomotion and hand function in patients with cerebral palsy (see supplementary table 1) ^{83,84}.

For cognitive ability we used a 3-point score: normal level of function, learning disabilities and severe cognitive impairment, as assessed by developmental milestones, school performance and occupation.

Magnetic resonance images of all patients were available for evaluation. The studies were evaluated according to a standard protocol ².

Genotypic inventory

Mutation analysis of *DARS2* was performed as described earlier ⁶. Several new primers were used, which are listed in supplementary table 2. The presence of the c.228-20T>C polymorphism on the same allele as another intron 2 mutation was determined by sequence analysis of the equivalent PCR fragment obtained from the parental DNA. If both parents carried the c.228-20T>C polymorphism, the patient's PCR fragment was cloned into a pGEM-T vector (Promega), which was then transformed into *E. coli* and individual clones were sequenced. Genotype data of all 68 patients of the Amsterdam database up to December 2012 and the 10 patients of the Helsinki database were documented. Additionally, the mutations of 42 published patients with two *DARS2* mutations were reviewed ^{15,57,58,80,81,85–87}.

Statistical analysis

Descriptive statistics were used to review the phenotypes and genotypes. To test whether there is an association between age of onset and disease progression in the first 10 years after disease onset, we performed a non-parametric resampling procedure with 100000 permutations to study the differences between the empirical cumulative distribution functions over the ordinal severity scale of consecutive age-of-onset groups. Cox regression analysis was performed to study the relation between the continuous covariate 'age of onset' and disease duration at the time of loss of ambulation. The recursive partitioning technique of Hothorn et al. ⁹⁷ was used to analyse whether categories of patients with different ages of onset could be formed. Kaplan-Meier curves were constructed to visualize the probabilities for the loss of the ability to walk without support and full wheelchair dependency relative to the duration of the disease, with a distinction for age of onset. For patients in whom the event of loss of walking without support or full wheelchair dependency had not yet occurred at the last clinical evaluation, the event was indicated as censored. The log rank test was used to compare subgroups. Student's t-test was used to assess the difference in the levels of *DARS2* intron 2 inclusion. Analyses were performed using the statistical software program R and SPSS for Windows version 20.

Cell culture

Control and patient-derived lymphoblasts were obtained and cultured as described previously ⁶⁵.

HEK293T cells were grown in OptiMEM (Invitrogen) and 10% fetal bovine serum. Cells were cultured at 37°C and 5% CO₂.

Enzyme assay

Mitochondria were isolated from lymphoblasts using the Mitochondrial Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Isolated mitochondria were re-suspended in reaction buffer (50 mM HEPES-KOH pH 7.6, 25 mM KCl, 12 mM MgCl₂ with protease inhibitors (Roche)). Mitochondrial extracts were obtained after incubation in 0.5% NP40 for 10 minutes and centrifugation at 13,000 revolutions per minute for 10 minutes. Mitochondrial extracts were run on a SDS-PAGE gel with 0.5% trichloroethanol and protein loading was analysed with the Gel Doc EZ system (Bio-rad). Aminoacylation assays were carried out in 50 mM HEPES-KOH pH 7.6, 25 mM KCl, 12 mM MgCl₂, 2.5 mM ATP, 0.2 mg/mL bovine serum albumin, 1 mM spermine, 32 µM stable-isotope labelled aspartate, 40 µM total *E. coli* tRNA (Roche) and mitochondrial extract at 37°C. 0.1 mM NaAc pH 5.2 was added to the samples at indicated time points to stop the reaction. tRNAs were isolated immediately, using acidic phenol/CHCl₃/IAA and ethanol precipitation. Aspartate was released from the tRNA by incubation in 62.5 mM borate buffer pH 10.0 for 1 hour at 42°C. Stable-isotope labelled aspartate was measured by liquid chromatography–tandem mass spectrometry.

Compound screen

For the compound screen, a splicing reporter construct was used, containing the most common mutation in LBSL patients (c.228-21_-20delTTinsC), transfected in HEK293T cells together with a pmCherry-N1 plasmid (Clontech) as control. Splicing reporter constructs contained exon 2, intron 2, exon 3, intron 3 and part of exon 4 of *DARS2* fused to enhanced yellow fluorescent protein (EYFP), as previously described⁵⁹. This reporter construct leads to expression of EYFP when exon 3 is skipped. Compounds from the Spectrum Collection (Microsource), a library of 2000 FDA approved and natural compounds, were screened for their effect on expression of the splicing reporter construct.

HEK293T cells were transfected with polyethylenimine (PEI, Polysciences) at 50% confluency. Cells were co-transfected with a pmCherry plasmid to control for the number of cells, transfection efficiency and possible other effects of the compounds on, for example, protein stability and cell viability. At an earlier stage, we had found that sodium orthovanadate increased correct splicing of the third exon of *DARS2*, and this compound was therefore used as a positive control. The stock solution of each compound was 100 µM dissolved in 1% dimethyl sulfoxide. Compounds were added 24 hours after transfections at a final concentration of 1 µM. After incubation for 24 hours, cells were fixed in 4% paraformaldehyde and EYFP and mCherry expression was measured on a Cellomics ArrayScan VTI HCS Reader. The screen was done in duplo. The 2000 screened compounds were ranked based on the EYFP / mCherry ratio with the rank product method⁸⁸.

The top five hits from this analysis were used for further validation on transfected HEK293 cells

in different concentrations, as indicated in the text and figures. The compounds that were used for validation were obtained from Sigma. These selected compounds were also used in combination with two additional constructs, one with intron 2 mutation c.228-20_-11delinsCCCCCCCCCG and the other with c.228-20_-15delinsCCCCCA, to demonstrate that the effects were not restricted to one single mutation, c.228-21_-20delTTinsC.

RNA isolation and quantitative PCR

Transfected cells were harvested 48 hours after transfection and 24 hours after addition of the compounds. Total RNA was extracted using TRIzol® (Invitrogen). First-strand cDNA synthesis was carried out with Superscript III RT (Invitrogen). PCR was done on complementary DNA (cDNA) with Platinum Taq according to the manufacturer's protocol (Invitrogen). Quantitative PCR (qPCR) was performed using SYBR® Green (Roche) on a LightCycler 480 (Roche). The primers are described in supplementary table 2.

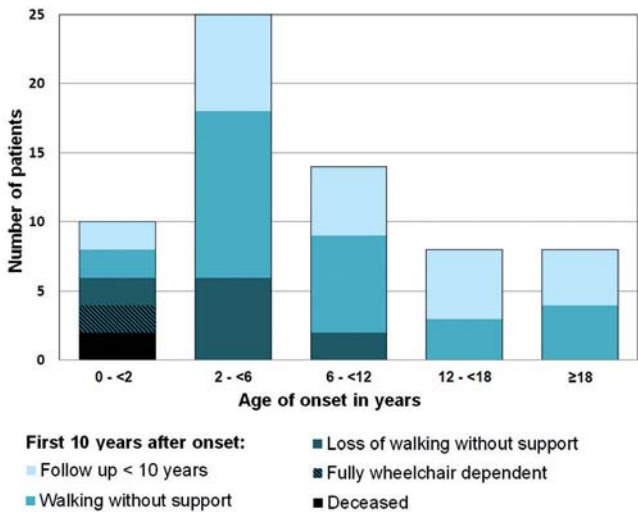


Figure 1 Age of onset and disease progression in the first 10 years. Plot of the number of LBSL patients per age category of disease onset, with a subclassification for disease progression in the first 10 years after disease onset. Patients not being followed for 10 years are represented in light blue, unless they were already categorized in the most severe groups (deceased or fully wheelchair dependent).

Results

Phenotypic spectrum

The databases contained 78 patients with two *DARS2* mutations. Patients for whom no clinical information could be collected ($n=11$) were excluded from the study. One patient was also excluded because of co-morbidity (serious hypoplasia of the right cerebellar hemisphere¹²). For some patients, the available clinical information was incomplete; these patients were only excluded from the analysis for the subject of the missing information. Sixty-six LBSL patients (36 female and 30 male patients, 58 families, see supplementary table 3) were available for the clinical inventory. The average age of the patients at the latest clinical evaluation was 24 years (standard deviation 14 years, range 1 - 59 years).

Age of onset: The mean age at which patients first showed neurological signs, was 8 years (standard deviation 8 years, median 5 years, range 0.4 - 40 years). In 53% of the patients, the disease

began before the age of 6 and in 88% before the age of 18 years (Figure 1). Onset in late teens was rare. Eight patients had adult onset, seven of whom were female. The most common neurological sign was cerebellar ataxia, especially gait ataxia. One patient (LBSL236) has thus far been asymptomatic. He had been referred for a neurological examination at the age of 3 months because of abnormal muscle tone. Cranial ultrasound examination revealed hyperechogenicity of both cerebral hemispheres and MRI showed abnormalities typical of LBSL, after which the diagnosis was confirmed by DNA analysis. He is presently a normal boy of 2 years old.

Early motor development: The majority of patients achieved unsupported walking at a normal age. Nine out of 63 patients showed a delayed development and three patients never achieved unsupported walking. Patients with a delayed early development had a more severe disease course.

Loss of ambulation: Only eight patients required an aid for walking before the age of 12 years and only four became fully wheelchair dependent before 12 years. Patients in their teens were more likely to have lost walking without support, but not to be fully wheelchair dependent. In patients of 18 years and older, 50% required support for walking and 13% was fully wheelchair dependent.

GMFCS and MACS: The majority of patients was able to walk without (GMFCS I-II) or with (GMFCS III) support. Most patients had no (MACS I) or limited (MACS II) problems with handling objects. Manual ability was generally less severely affected than ambulation. It was exceptional for patients to score on the highest level V of both systems, corresponding to complete wheelchair dependency and a severely limited manual ability. This was only the case in a small number of patients with disease onset before 2 years and rapid deterioration. Two middle aged patient are currently also severely handicapped (GMFCS IV or V and MACS IV).

Cognitive ability: Most patients had a normal learning ability, while 20% (12/62) required special education. Serious intellectual disability was observed in only two patients.

Mortality and survival: In this cohort of 66 patients, 15 individuals had reached the age of at least 35 years. Only two patients had died. Both had infantile disease onset, rapid disease progression and death before the age of two years.

MRI characteristics: Except for three patients, all patients in the databases fulfilled all major and one or more minor MRI criteria for LBSL⁸². Two siblings (LBSL160/161) and one other patient (LBSL223) lacked the major criterion of signal abnormalities throughout the pyramidal tracts: there was no involvement of the pyramids at the medulla oblongata and the lateral corticospinal tracts of the spinal cord. LBSL223 lacked abnormalities in cerebellum and brain stem at the age of two years (Supplementary Figure 1). One patient (LBSL229) showed additional signal abnormalities in the anterior funiculus over the entire length of the spinal cord. In general, in late onset, mildly affected patients, the cerebral white matter abnormalities were less profound than in severely affected patients (Supplementary Figure 2).

Correlation between age of onset and disease severity: For the comparison between age of onset and disease severity after 10 years, 42 patients were available. We divided the patients in age of onset categories that are in common use in medicine: infantile (0 - <2 years), early juvenile (2 - <6 years), late juvenile (6 - <12 years), teenage (12 - <18 years), and adolescent and adult (18 years

and older). When comparing these patient categories, earlier onset was related to a more severe neurological deterioration in the first 10 years after disease onset ($p = 0.00011$; Figure 1).

When studying the relation between the continuous covariate age of onset and the time at loss of ambulation, only the time that patients became fully wheelchair dependent correlated with the age of onset (cox regression analysis, $p = 0.014$). Based on the time at full wheelchair dependency, evidence was found for the categorization into two groups of patients (age of onset 0 - 1.5 years and age of onset >1.5 years; $p = 0.022$). Patients with an infantile onset lost the ability to walk without support ($p = 0.0001$) soonest after disease onset and became fully wheelchair dependent sooner ($p < 0.0001$; Figure 2). All the adult onset patients were still ambulant, without or with support, at the last clinical evaluation.

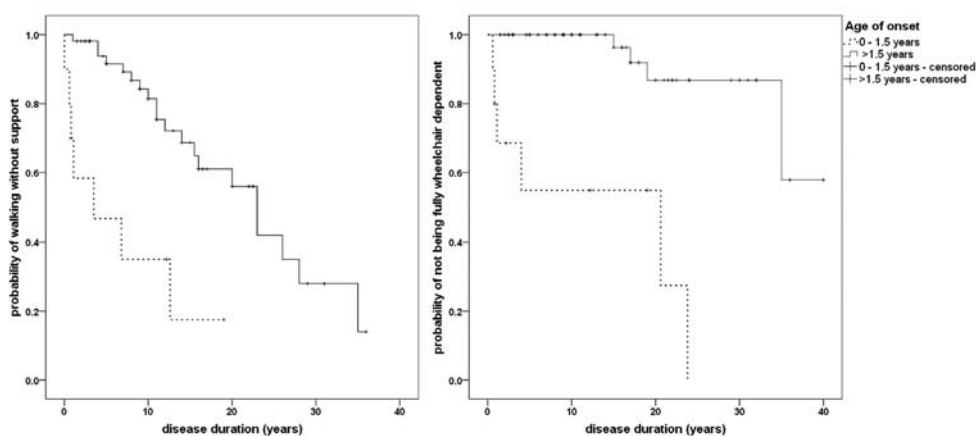


Figure 2 Loss of ambulation in relation to age of onset. Kaplan-Meier plots showing the probability of walking without support (left panel) and not being fully wheelchair dependent (right panel) in relation to disease duration for patients with an onset before or after the age of 1.5 years, respectively.

Genotypic variation

Since the discovery of *DARS2* mutations in 38 LBSL patients in 2007⁶, the diagnosis of LBSL has been confirmed by DNA analysis in our laboratories in 40 additional patients (39 families). Of the mutations found, 13 have not been published before. In addition, 42 patients with two pathogenic mutations have been published by others^{15,57,58,80,81,85-87}.

An overview of all 60 known mutations is displayed in Figure 3 and the mutations of our patients are listed in Supplementary Table 3. The mutations are spread over the entire *DARS2* gene (Figure 3). Ninety-four percent (113/120) of the patients had one intron 2 mutation in the polypyrimidine tract just upstream of exon 3. Thirteen different mutations were found in this region with c.228-21_-20delTTinsC being the most common (88/120). In all Amsterdam database patients, these intron 2 mutations co-segregated with a single nucleotide polymorphism (c.228-20T>C) on the same allele, which resulted in a modified description of some earlier published mutations (Supplementary Table 3). All patients in our database were compound

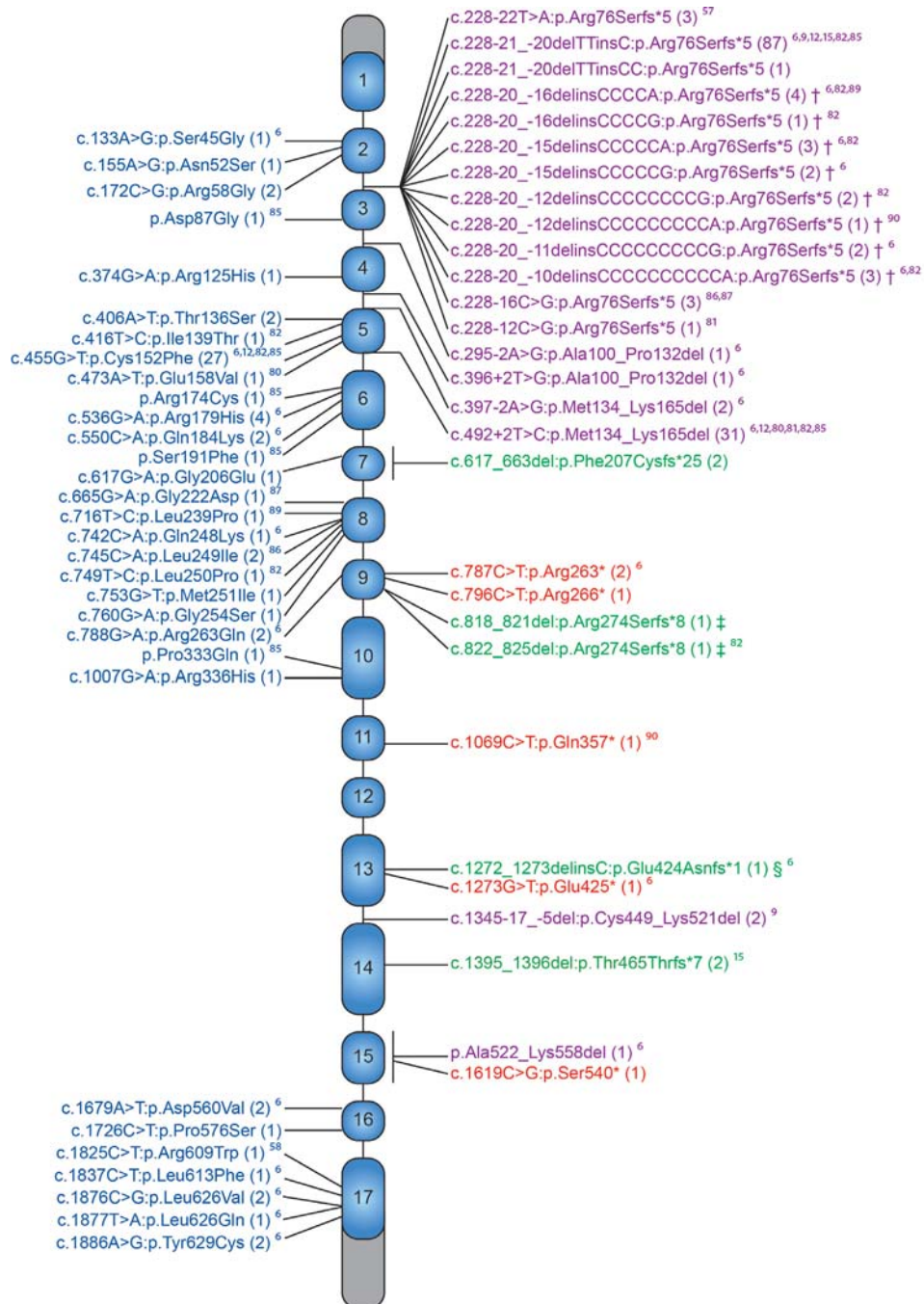


Figure 3 DARS2 mutations. Schematic representation of the DARS2 gene and the distribution and frequencies (between brackets) of patients in our databases and all published mutations. The exons are displayed in proportion, the introns are shortened. Splice site mutations are indicated in purple, missense mutations in blue, deletions in green, and nonsense mutations in red. † mutation was previously published with a different annotation; ‡ p.Arg274Serfs*9 in current HGVS nomenclature; § p.Glu424Asnfs*2 in current HGVS nomenclature.

heterozygous. Four patients (two families) have been described with homozygous mutations^{57,58}. In one patient (LBSL44) three mutations were found that were predicted to be pathogenic. For this patient, no parental DNA was available to investigate the allelic distribution.

Genotype-phenotype relation

Of 66 phenotyped LBSL patients, 25 patients had a private mutation and 31 patients had a unique combination of mutations. The fact that only 22 patients had a combination of *DARS2* mutations shared by other unrelated patients hampered the study of a genotype-phenotype correlation.

In the 45 patients who had the common c.228-21_-20delTTinsC mutation and different mutations on the second allele, the clinical severity ranged from childhood onset disease with loss of ambulation before adulthood to very slowly progressive adult onset disease. There was no evident relationship between the location or type of the second mutation and disease severity. Interestingly, the four patients with the most severe phenotype did not have the common mutation, but had another intron 2 mutation.

Two groups could be formed with multiple patients sharing the same combination of mutations: c.228-21_-20delTTinsC together with c.455G>T ($n=9$) or with c.492+2T>C ($n=11$). All patients in these groups had a benign phenotype characterized by an onset in childhood up to young adulthood followed by mildly progressive neurological deterioration. There was no significant difference in clinical outcome between these groups or between these groups and the rest of the patients. Two additional patients with the same genotype (LBSL103 and LBSL166, c.228-21_-20delTTinsC, c.397-2A>G) had a mild disease with an adult onset.

Only four patients did not have an intron 2 mutation affecting the splicing of exon 3. This number was too small to conclude whether their phenotype is different. It was striking however that two of these patients (LBSL160/161) lacked some major MRI criteria, as described above. Another patient who lacked some of the classical MRI characteristics (LBSL223) had a unique intron 2 mutation and a rather severe disease course.

In seven of the eight affected sibling pairs, the first symptoms occurred within the same phase of life for both patients, with a maximum difference of three years; in one patient (HEL3) the symptoms occurred 13 years later than in his brother (HEL2). Among five affected sibling pairs, mild differences in clinical severity of motor dysfunction or cognitive problems were observed.

Decreased mitochondrial aspartylation in LBSL patients

Mitochondrial aspartyl-tRNA synthetase activity was measured in mitochondrial extract from lymphoblasts of four LBSL patients (LBSL8, LBSL44, LBSL160 and LBSL266) and four controls. The patients had different combinations of mutations and their phenotypes ranged from infantile onset disease with severe neurological deterioration (LBSL266) up to adult onset, mild disease (LBSL44) (Supplementary Table 3). The results show a substantial loss of activity of mitochondrial aspartyl-tRNA synthetase in all patient cells compared to controls (Figure 4

and Supplementary Figure 3). The level of residual enzyme activity was in the same range for all patients with overlap in values for the most severely and mildest affected patients.

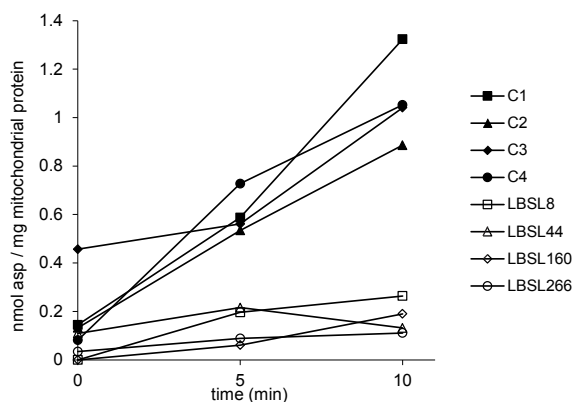


Figure 4 Mitochondrial aspartyl-tRNA synthetase activity in lymphoblasts. The aspartylation activity in isolated mitochondria from four control and four patient lymphoblasts was measured as described in the methods section. Activity was strongly reduced in patient lymphoblasts (LBSL8, LBSL44, LBSL160 and LBSL266) as compared to control lymphoblasts (C1-C4).

Compounds altering *DARS2* exon 3 splicing

We have previously developed a yellow fluorescent protein (YFP) reporter construct, in which increased splicing efficiency of intron 2 is reflected by a decrease in the YFP signal⁵⁹. These results were validated at both the RNA and protein level. In the present study, we used this construct with the most common splice site mutation to screen compounds for their ability to modify the splicing efficiency of intron 2. Of the 2000 compounds ranked by the rank product method, the five compounds causing the largest decrease in the EYFP/mCherry ratio in both screens were used for further studies: gentian violet, cantharidin, pyrithione zinc, celastrol and alanyl-dl-leucine (Supplementary Table 4). Effects of all compounds except for alanyl-dl-leucine could be confirmed in separate experiments using the reporter construct. The two additional reporter constructs with different splice site mutations in the same region showed similar effects (Figure 5A). So, the identified compounds affect the splicing efficiency at the intron 2/exon 3 boundary independent of the exact mutation.

To directly assess the effect on mRNA splicing, reverse transcriptase (RT) PCR was performed to detect the mRNA with and without the third exon of *DARS2*. In addition to sodium orthovanadate, one of the selected compounds, cantharidin, showed a direct effect on the splicing of the third exon. Both compounds increased the amount of correctly spliced product (Figure 5B). There was a concentration-dependent effect up to 10 μ M for cantharidin (Figure 5C). In experiments with a smaller number of different concentrations, we showed that the concentration-dependent effects were reproducible (Supplementary Figure 4A) and all three mutant reporter constructs respond in a similar concentration-dependent manner (Supplementary Figure 4B).

Cantharidin had a clear effect on splicing of intron 2 on the mRNA level (Figure 5B) and was chosen to study the effect on lymphoblasts from LBSL patients. Two patient-derived lymphoblast cell lines and two control cell lines were treated with 10 μ M cantharidin for 24 hours. Quantitative RT-PCR on RNA purified from these cells showed that cantharidin increased the correct splicing

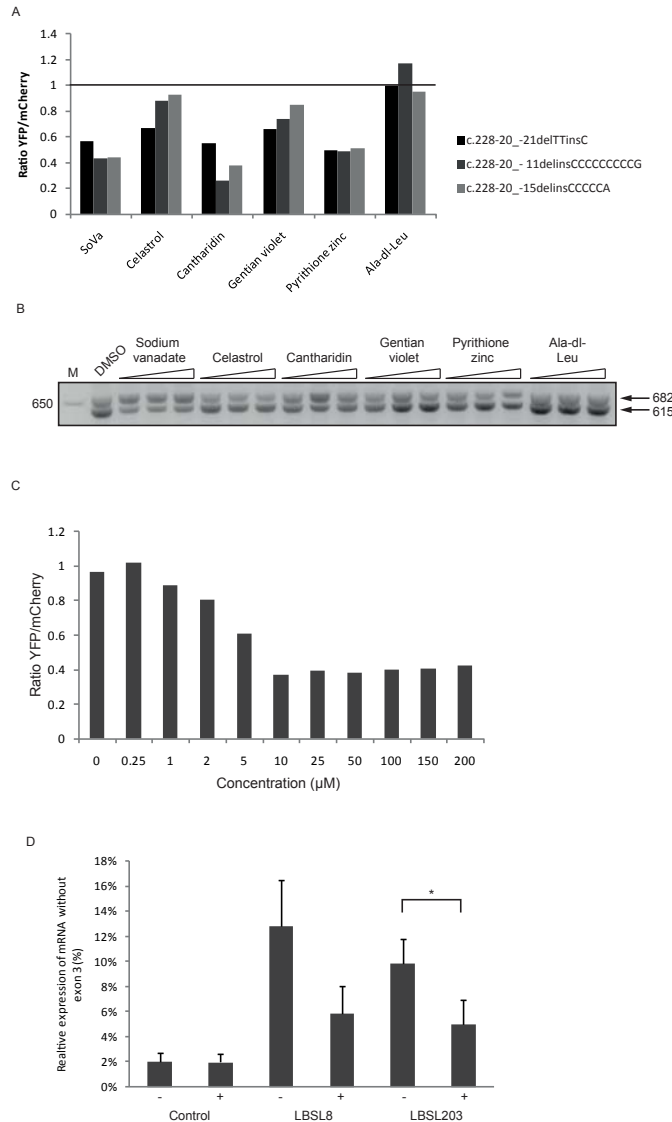


Figure 5 Compounds affecting DARS2 exon 3 splicing. (A) The effects of selected compounds on three different reporter constructs are shown. The effect of DMSO was set at a YFP / mCherry-ratio of 1. This ratio is decreased with all selected compounds except Alanyl-dl-leucine. The three different mutations in the reporter constructs show similar results. (B) RT-PCR was performed on cells transfected with the reporter construct and treated with the indicated compound. After isolation of total RNA and cDNA synthesis, the inclusion or exclusion of exon 3 was visualized by PCR with primers 2Fb and GFP-R (Supplementary Table 1) followed by agarose gel electrophoresis. The expected sizes of the bands are 615 bp (without exon 3) and 682 bp (with exon 3) as indicated on the right. M represents a marker lane containing the Invitrogen 1Kb Plus DNA ladder and the size of the band is indicated on the left. (C) The effect of increasing concentrations of cantharidin on the YFP / mCherry-ratio is shown. (D) Relative expression of mitochondrial aspartyl-tRNA synthetase mRNA without exon 3 in control and patient lymphoblasts with and without treatment of 10μM cantharidin for 24 hours. The expression of the variant without exon 3 is shown as the mean percentage of total amount of mitochondrial aspartyl-tRNA synthetase mRNA \pm standard error of the mean. The asterisk indicates $p < 0.05$.

of *DARS2* exon 3 in these patient-derived cells. With the addition of cantharidin, the percentage of mRNA without exon 3 was decreased on average from 11.3% to 5.4% (Figure 5D).

Discussion

In this study, we investigated the phenotypic variation in a relatively large number of LBSL patients. LBSL was originally described as a juvenile onset disorder with a reliable, slow progression³. Our study confirms that LBSL is a neurological disorder with generally slow progression and low mortality, with in some cases even later onset and slower disease course than initially described. Complete wheelchair dependency is rare, especially before the age of 18 years. Life expectancy may be normal for most patients, as the oldest patient in this cohort is 59 years old and in quite a good condition. Altogether, LBSL patients have in general a better prognosis than most other hereditary leukoencephalopathies. The infantile onset cases are an exception. They form a distinct category characterized by a more rapid neurological deterioration and in the most severe cases early death. The implication of our observations is that both pediatric and adult neurologists may meet patients with LBSL and need to be aware of this disease.

The patients included in the present study had been selected for analysis of the *DARS2* gene on the basis of specific MRI findings. A limitation to this approach is that, possibly, LBSL patients with an atypical MRI pattern and asymptomatic patients have been missed. So far, one index patient (LBSL236) and three siblings of LBSL patients with the same pathogenic mutations are known that are asymptomatic⁸⁶ (personal observations). We suspect that until now most early-onset patients have remained undiagnosed, because the severe phenotype is not widely known. Consequently, the clinical spectrum of the disease may still be wider and the distribution over ages of onset may be different than currently described.

Almost all patients with LBSL have compound heterozygous *DARS2* mutations. Mitochondrial aspartyl-tRNA synthetase is an enzyme that is essential for life and patients never have two null mutations. These observations suggest that the range of permissive mutations, resulting in mitochondrial aspartyl-tRNA synthetase activity that is high enough not to be lethal, but below a certain threshold for symptoms to appear, is narrow. The c.228-21_-20delTTinsC mutation has not been reported in the homozygous state in any LBSL patient, which is striking considering the relatively frequent occurrence of this mutation in LBSL patients, and the high carrier rate of 1:95 that has been described in the Finnish population¹². It is also striking that there is no LBSL patient who is compound heterozygous for two different intron 2 splice site mutations. The intron 2 splice site mutations are leaky and allow the production of some normal protein. It is possible that the presence of an intron 2 splice site mutation on both alleles leads to another pathology, but we suspect that this may not lead to a disease. We postulate that the remaining mitochondrial aspartyl-tRNA synthetase activity in patients' cells is largely derived from the allele with the 'leaky' intron 2 mutation, while the second mutation probably is a functional null allele. One patient (LBSL44) had three predictively pathogenic mutations; it is most likely that the intron 2 mutation is located on one allele, and the two other mutations on the other allele, leading to one null allele.

We found that a known single nucleotide polymorphism (c.228-20T>C) invariably co-segregates with mutations in the polypyrimidine tract in intron 2 in patients from the Amsterdam database. It is unknown whether this is also the case in other patients. Our finding raises the possibility that a single mutation in this intron 2 tract may only be pathogenic when occurring in combination with this single nucleotide polymorphism.

Unfortunately, the heterogeneous nature of the genotypes seriously hampered the genotype-phenotype correlation study. For a proper genotype-phenotype correlation study much larger groups of patients are required. Interestingly, four severe patients do not have the common c.228-21_-20delTTinsC mutation seen in 45 milder patients. One of these patients (LBSL263) has the same mutations as a patient described in the literature who was also severely affected⁸¹ (the presence of the above-mentioned single nucleotide polymorphism was not mentioned). Two groups of patients with similar mutations and a mild disease course could be formed, suggesting that these genotypes are related to a benign phenotype. Striking intrafamilial differences were absent. Outside our study, only one family has been described with three affected siblings⁵⁷ and these displayed remarkable interindividual differences. Our observations support the hypothesis that there is a genotype-phenotype correlation. Intrafamilial differences are most likely explained by the influence of environmental and other genetic factors.

We developed an enzyme assay to assess mitochondrial aspartyl-tRNA synthetase activity and for the first time confirmed its decreased activity directly in patient cells. Decreased activity of mutant enzymes has been shown before in *in vitro* aminoacylation assays from overexpression studies^{6,91}, but it is difficult to extrapolate these results to patient cells, where the activity is virtually always determined by a combination of two different mutant alleles. We demonstrate that the tested patient cells have a significantly reduced mitochondrial aspartyl-tRNA synthetase activity. We found no clear correlation between enzyme activity and severity of the phenotype. Possibly, the assay we used was not sensitive enough to detect subtle differences. It could also be that lymphoblasts are not the most suitable cells to show the differences; they might be better detectable in other cell types, like neuronal cells.

The very high occurrence of splice site mutations makes the splicing process a promising target for therapy. We have previously demonstrated that antisense oligonucleotides can alter the splicing efficiency at the intron 2/exon 3 boundary⁵⁹. Despite the great progress in the field of antisense oligonucleotides in the recent years, development of a therapy based on the successful delivery of antisense oligonucleotides to the central nervous system will probably not be possible through systemic administration⁹². Therefore, we used a compound library containing FDA-approved and natural compounds, many of which are known to be able to cross the blood-brain barrier. With this library, a screen was performed to find compounds that influence the intron 2/exon 3 splicing event. After validation of hits from this screen, cantharidin was identified as the most effective compound. It increased the inclusion of exon 3 in our reporter constructs and in lymphoblasts from LBSL patients. Cantharidin is a protein phosphatase 1 and 2A inhibitor. It has previously been found to increase exon 7 inclusion in the *SMN2* gene, relevant for spinal muscular atrophy⁹³. Reducing protein phosphatase 1 activity promotes usage

of numerous alternative exons, indicating that protein phosphatase 1 activity plays a role in splice site selection ⁹³. Cantharidin is too toxic for use in patients ⁹⁴, but this study provides proof-of-concept that influencing the splice site mutations is possible and a highly promising therapeutic target for LBSL. Future research should be directed at less toxic variants of cantharidin ⁹⁵ or other protein phosphatase 1 or 2A inhibitors.

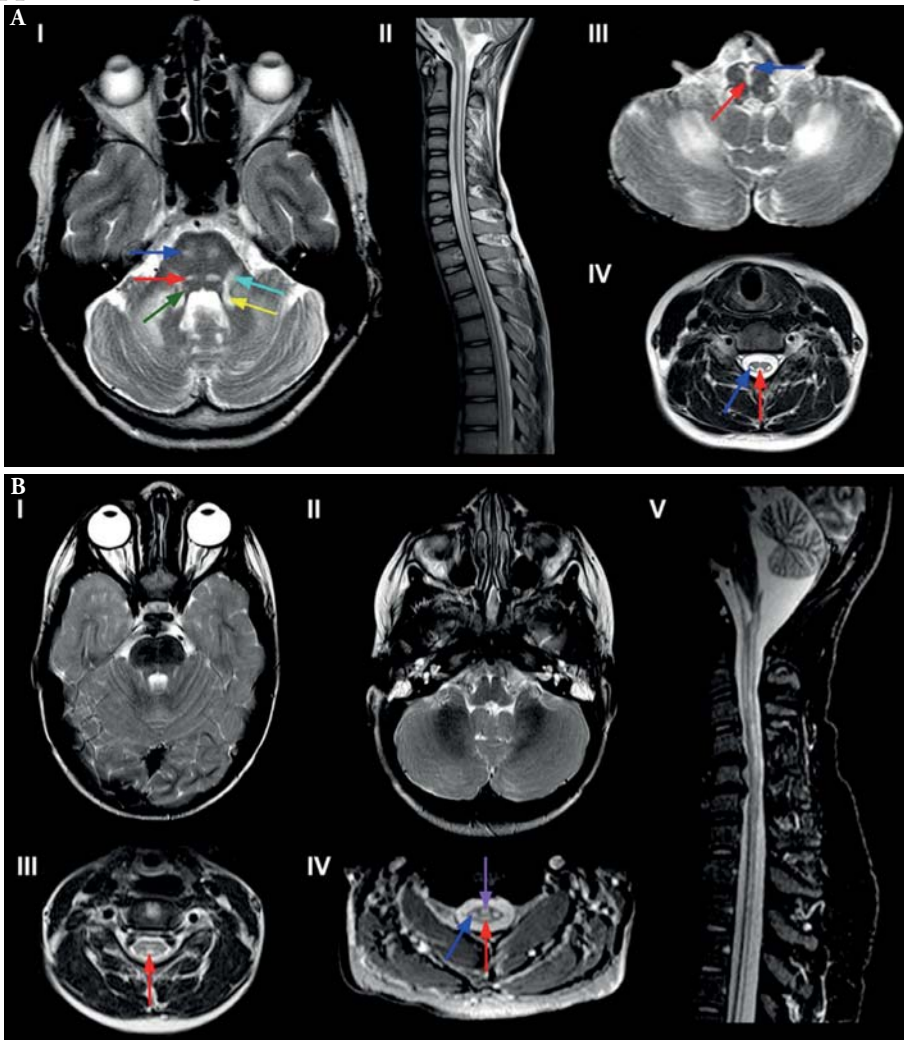
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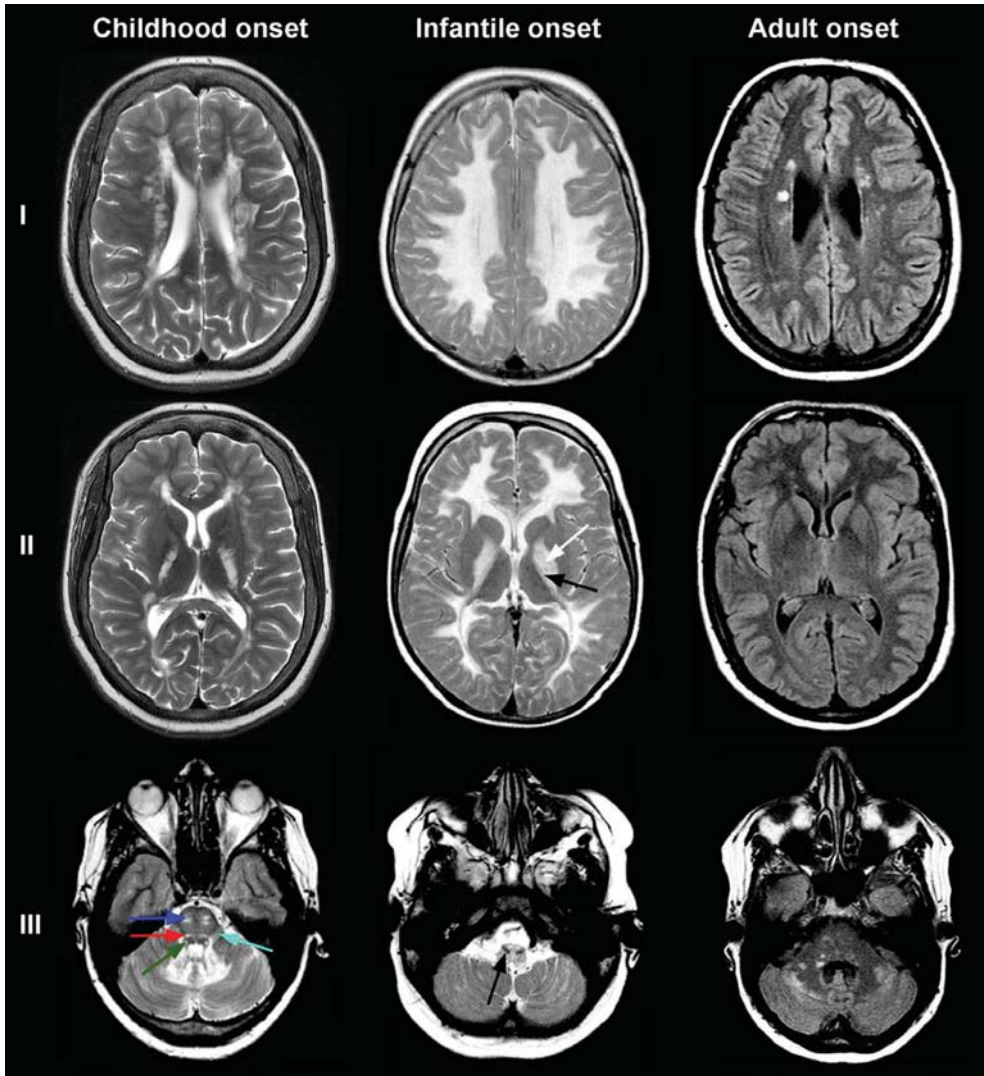
Funding

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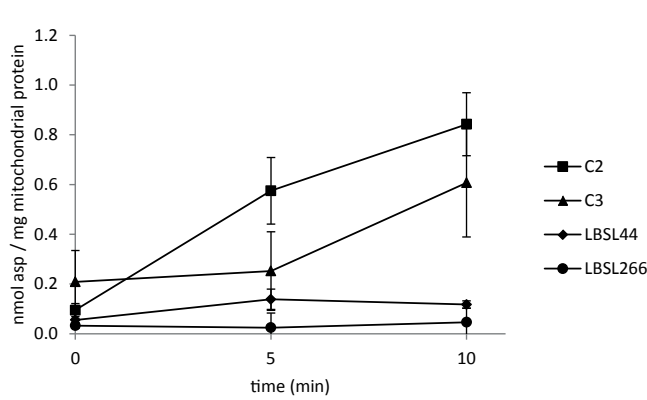
Supplementary figures and tables



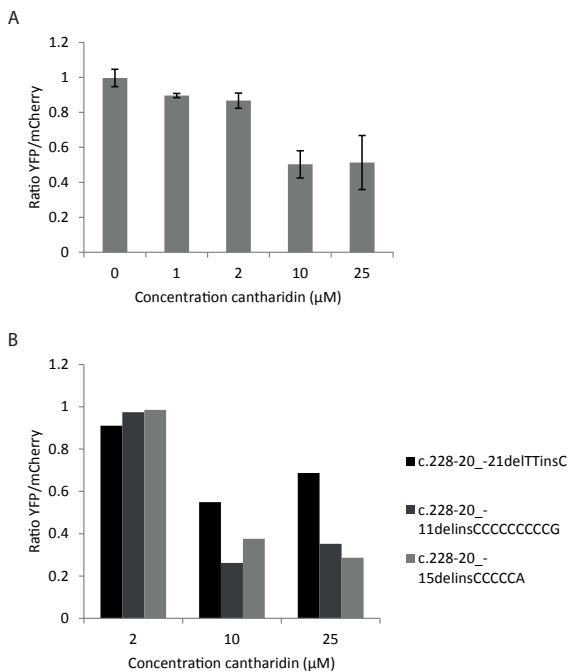
Supplementary Figure 1 Brain stem and spinal cord abnormalities in LBSL patients. (A) Typical MRI findings. At the level of the brain stem (I), an abnormal signal is seen in the medial lemniscus (red arrow), pyramidal tracts (blue arrow), intraparenchymal part of the trigeminal nerve (light blue arrow), mesencephalic trigeminal nerve tracts (green arrow) and superior cerebellar peduncles (yellow arrow). The subcortical cerebellar white matter is affected (III) and there are signal abnormalities throughout the spinal cord (II). The axial images show hyperintensity of the decussation of the medial lemniscus (red arrow) and pyramids (blue arrow) at the level of the medulla (III) and involvement of the dorsal columns (red arrow) and lateral corticospinal tracts (blue arrow) in the spinal cord (IV). (B) Atypical MRI findings. In LBSL223, there are no abnormalities in the medial lemniscus and pyramidal tracts at the level of the brain stem (I; major criteria). There are no cerebellar white matter abnormalities (II; minor criterion). The dorsal columns are affected (red arrow), but the major criterion of signal abnormalities in the lateral corticospinal tracts is missing (III). In LBSL229, the spinal cord is affected over the entire length (V), the axial image (IV) shows abnormalities in the dorsal columns (red arrow), lateral corticospinal tracts (blue arrow) and the anterior funiculus (purple arrow).



Supplementary figure 2 Variable extensiveness of MRI abnormalities. On the left, T2-weighted images of a classical, 20 year old LBSL patient (LBSL16) are shown. The periventricular white matter (I), posterior limb of the internal capsule, splenium of the corpus callosum (II), medial lemniscus (red arrow), pyramidal tracts (blue arrow), intraparenchymal part of the trigeminal nerves in the brain stem (light blue arrow), mesencephalic trigeminal nerve tracts (green arrow) and the cerebellar white matter have an abnormal signal (III). In the middle, T2-weighted images of a severe, infantile onset case (LBSL266) at the age of five years are shown. The cerebral white matter is diffusely affected (I and II) and the posterior limb of the internal capsule (black arrow) and globus pallidus (white arrow) are abnormal (II). In the medulla, a diffuse hyperintense signal is seen; the inferior olives (black arrow) are the only structures not affected (III). The FLAIR images on the right are from a 40 year old patient with mild disease (LBSL229). They show small, multifocal white matter lesions around the ventricles (I) and in the cerebellum (III). At the level of the basal ganglia the white matter is normal (II). The multifocal lesions in this patient are suggestive of Multiple Sclerosis, but the combination with the abnormalities in brain stem and spinal cord (Supplementary Figure 1B) is distinctive for the diagnosis LBSL.



Supplementary Figure 3
Reproducibility of mitochondrial aspartyl-tRNA synthetase activity measurements in control and patients' lymphoblasts. The aspartylation activity in isolated mitochondria from two control and two patient lymphoblast cell lines was measured as described in the methods section. The means of three separate experiments \pm standard deviation are shown.



Supplementary Figure 4 Cantharidin and DARS2 exon 3 splicing. (A) The effect of different concentrations of cantharidin on the reporter construct containing the c.228-21_-20delTTinsC is shown. The YFP / mCherry-ratio in the presence of DMSO was set at 1. Values are means of three separate experiments \pm standard deviation. (B) The effect of varying concentrations of cantharidin, as indicated below the figure, was tested on three different reporter constructs containing intron 2 mutations.

Supplementary Table 1 Overview of the five levels of the GMFCS and MACS (adapted from ⁹⁶)

| | GMFCS ⁸⁴ | MACS ⁸³ |
|-----------|--|---|
| | Motor function | Manual ability |
| LEVEL I | Walks without limitations | Handles objects easily and successfully |
| LEVEL II | Walks with limitations | Handles most objects but with somewhat reduced quality and/or speed of achievement |
| LEVEL III | Walks using a hand-held mobility device | Handles objects with difficulty; needs help to prepare and/or modify activities |
| LEVEL IV | Self-mobility with limitations; may use powered mobility | Handles a limited selection of easily managed objects in adapted situations |
| LEVEL V | Transported in a manual wheelchair | Does not handle objects and has severely limited ability to perform even simple actions |

Supplementary Table 2 Primers

| Primers for sequencing | | | |
|------------------------|-------|-------------|--|
| Oligo name | Exon | Orientation | Sequence |
| DARS2-Ex5F | 5 | Forward | GCTTAAGTGATCCTCCTGTCT |
| DARS2-Ex5R | | Reverse | TTCATGATGTGTCTACAATAAAATGC |
| DARS2-Ex6F | 6 | Forward | GTAAAACGACGGCCAGCAGTGGGCTACTTAATGATGAAAC |
| DARS2-Ex6R | | Reverse | CAGGAAACAGCTATGAGCAACATCTTGACCTCATGC |
| DARS2-Ex7F | 7 | Forward | TGGTAAAACGACGCCGACGAAGCCTCAGATTTGTTGTATA |
| DARS2-Ex7R | | Reverse | GTGTCTTGGCAGTAAATAAAAGTGGACCAAG |
| DARS2-Ex8F | 8 | Forward | GCAGGAAATTGTCTCTGTTCATTG |
| DARS2-Ex8R | | Reverse | AATCCCCTCTCACATCTACTACC |
| DARS2-Ex10F | 10 | Forward | GGCCATTAGCACAGTGTCTG |
| DARS2-Ex10R | | Reverse | TTGCTCTAGCTCTGTAACAACG |
| DARS2-Ex11F | 11 | Forward | TCATATTGCTTAACCCATGGTAA |
| DARS2-Ex11R | | Reverse | GCCACCACGCCTGACTAAT |
| DARS2-Ex13F | 13 | Forward | GCACAGAACTGGCACAGCTA |
| DARS2-Ex13R | | Reverse | ATGCAGAGCAGCTCCATTTT |
| DARS2-Ex15F | 15 | Forward | CCCGTAGAACAGAAAACCAGA |
| DARS2-Ex15R | | Reverse | GCAACAACAACAACAACACAA |
| cDARS2-1Fa | 1-3 | Forward | GAGAGTGGGAACTCCTGGAA |
| cDARS2-1Ra | | Reverse | TTGAACAAGCCCATCGAAAT |
| cDARS2-1Fb | 1-6 | Forward | TGTTGCAGAGTTCACAGAGGA |
| cDARS2-1Rb | | Reverse | GGACCTCAGTCGCAGGTTAT |
| cDARS2-3Fb | 10-15 | Forward | TGACTTTTGCTGAGGTGCTG |
| cDARS2-3Rb | | Reverse | TGCAGCTCTGCATTGTGAAT |
| cDARS2-4F | 14-17 | Forward | GGAGGAAAATCCCAGAGAGC |
| cDARS2-4R | | Reverse | AAAATCCAAATGATGCATGAAA |

| Primers for qPCR and RT-PCR | | |
|-----------------------------|-------------|-------------------------|
| Oligo name | Orientation | Sequence |
| 2F | Forward | ACCAACACATGTGGAGAGTTGCG |
| 3/4R | Reverse | TGCCTTCGGTACTGAATCCATCC |
| 2/4R | Reverse | GAGGCTGCCGACTTCGGTACTGA |
| 2Fb | Forward | ACCATGGCATGTGGAGAGTTGCG |
| GFP-R | Reverse | GTTGTGGCGGATCTTGAAGT |

Supplementary Table 3. Overview of phenotype and genotype LBSL patients

| Patient | Family | Gender | Age ^a | Mutation 1 ^b | Amino acid change | Mutation 2 ^b | Amino acid change | Unsup-ported walking | First neu- rological signs | Loss of un- supported walking | Loss of sup- ported walking | GMFCS ^c | MACS ^d | Learning ability ^e | Death |
|---------|---------|--------|------------------|-------------------------|-----------------------|-------------------------|-------------------|----------------------|----------------------------------|-------------------------------------|--------------------------------------|--------------------|-------------------|----------------------------------|-------|
| 1 | LBSL213 | 1 | f | 18 yrs | c.155A>G | | p.Asn52Ser | p.Cys152Phe | 18 mo | 2 yrs | - | II | I | nl | - |
| 2 | LBSL16 | 2 | f | 27 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Ala522_Lys558del | 15 mo | 5 yrs | 17 yrs | III | II | ↓ | - |
| 3 | LBSL222 | 3 | m | 16 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Arg336His | 14 mo | 6 yrs | - | I | I | ↓ | - |
| 4 | LBSL147 | 4 | m | 19 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys449_Lys521del | 11 mo | 13 yrs | 17 yrs | III | I | nl | - |
| 5 | LBSL72 | 5 | f | 59 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Asp560Val | nl | 27 yrs | 53 yrs | III | II | nl | - |
| 6 | LBSL73 | 5 | f | 51 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Asp560Val | 13 mo | 27 yrs | 47 yrs | III | I | nl | - |
| 7 | LBSL2 | 6 | m | 29 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Leu626Val | 14 mo | 6 yrs | - | I | I | nl | - |
| 8 | LBSL187 | 7 | f | 43 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Arg125His | 12 mo | 12 yrs | - | II | II | nl | - |
| 9 | LBSL166 | 8 | f | 37 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Met134_Lys165del | 24 mo | 30 yrs | 31 yrs | II | I | nl | - |
| 10 | LBSL103 | 9 | f | 31 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Met134_Lys165del | 11 mo | 20 yrs | - | II | I | nl | - |
| 11 | LBSL269 | 10 | f | 11 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Ile139Thr | 9 mo | 6 yrs | 11 yrs | II | I | nl | - |
| 12 | LBSL168 | 11 | f | 21 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys152Phe | 11 mo | 6 yrs | - | I | I | nl | - |
| 13 | LBSL80 | 12 | m | 25 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys152Phe | 14 mo | 23 yrs | - | - | - | nl | - |
| 14 | LBSL81 | 12 | f | 23 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys152Phe | 15 mo | 20 yrs | - | - | - | nl | - |
| 15 | LBSL21 | 13 | m | 24 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys152Phe | nl | 14 yrs | - | - | - | nl | - |
| 16 | LBSL36 | 14 | f | 12 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys152Phe | 11 mo | 2 yrs | - | I | I | nl | - |
| 17 | LBSL79 | 15 | m | 20 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys152Phe | 15 mo | 11 yrs | 19 yrs | III | II | nl | - |
| 18 | LBSL236 | 16 | m | 2 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys152Phe | 14 mo | not yet | - | I | I | nl | - |
| 19 | HEL8 | 17 | f | 8 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys152Phe | 14 mo | 3 yrs | - | I | I | nl | - |
| 20 | HEL9 | 18 | m | 5 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Cys152Phe | 38 mo | 2 yrs | - | II | I | - | - |
| 21 | LBSL28 | 19 | m | 29 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Met134_Lys165del | 14 mo | 1 yrs | 8 yrs | IV | III | nl | - |
| 22 | LBSL185 | 20 | f | 25 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Met134_Lys165del | 14 mo | 3 yrs | 19 yrs | III | II | nl | - |
| 23 | LBSL45 | 21 | f | 24 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Met134_Lys165del | 14 mo | 3 yrs | 12 yrs | II | II | ↓ | - |
| 24 | LBSL20 | 22 | m | 19 yrs | c.228-21_-20delTTinsC | | p.Arg76Serfs*5 | p.Met134_Lys165del | - | 2 yrs | 16 yrs | III | I | nl | - |

| Patient | Family | Gender | Age ^a | Mutation 1 ^b | Amino acid change | Mutation 2 ^b | Amino acid change | Unsup-ported walking | First neu- rological signs | Loss of un- supported walking | Loss of sup- ported walking | GMFCS ^c | MACS ^d | Learning ability ^e | Death | |
|---------|---------|--------|------------------|-------------------------|---|-------------------------|-------------------|------------------------------|----------------------------|-------------------------------|-----------------------------|--------------------|-------------------|-------------------------------|-------|---|
| 25 | HEL1 | 23 | f | 39 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.492+2T>C | p.Met134_Lys165del | 18 mo | 10 yrs | 20 yrs | - | III | II | nl | - |
| 26 | HEL2 | 24 | m | 25 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.492+2T>C | p.Met134_Lys165del | 11 mo | 2 yrs | 24 yrs | - | II | I | nl | - |
| 27 | HEL3 | 24 | m | 22 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.492+2T>C | p.Met134_Lys165del | 10 mo | 15 yrs | - | - | I | I | nl | - |
| 28 | HEL4 | 25 | f | 20 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.492+2T>C | p.Met134_Lys165del | 15 mo | 3 yrs | - | - | II | II | nl | - |
| 29 | HEL5 | 26 | f | 14 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.492+2T>C | p.Met134_Lys165del | 15 mo | 9 yrs | - | - | I | I | nl | - |
| 30 | HEL6 | 27 | m | 18 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.492+2T>C | p.Met134_Lys165del | 12 mo | 10 yrs | - | - | II | I | ↓ | - |
| 31 | HEL10 | 28 | f | 59 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.492+2T>C | p.Met134_Lys165del | 13 mo | 9 yrs | 44 yrs | IV | IV | - | - | - |
| 32 | LBSL44/ | 29 | f | 52 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.536G>A | p.Arg179His | 22 yrs | 45 yrs | - | III | I | nl | - | - |
| 33 | LBSL107 | 30 | m | 50 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.536G>A | p.Arg179His | nl | childhood | 45 yrs | - | III | II | nl | - |
| 34 | LBSL108 | 30 | f | 46 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.536G>A | p.Arg179His | nl | childhood | - | - | II | I | nl | - |
| 35 | LBSL90 | 31 | m | 22 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.550C>A | p.Gln184Lys | 13 mo | 6 yrs | - | - | I | I | nl | - |
| 36 | LBSL76 | 31 | m | 20 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.550C>A | p.Gln184Lys | 12 mo | 7 yrs | 18 yrs | - | III | III | ↓ | - |
| 37 | LBSL152 | 32 | f | 13 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.617_663del | p.Phe207Cysfs*25 | 21 mo | 0.8 yrs | - | - | II | III | nl | - |
| 38 | LBSL199 | 33 | m | 12 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.617G>A | p.Gly206Glu | 14 mo | 3 yrs | - | - | I | II | nl | - |
| 39 | LBSL91 | 34 | m | 19 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.742C>A | p.Gln248Lys | 10 mo | 2 yrs | - | - | II | I | nl | - |
| 40 | LBSL7 | 35 | m | 20 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.787C>T | p.Arg263* | 24 mo | 2 yrs | 6 yrs | - | II | II | ↓ | - |
| 41 | LBSL8 | 35 | f | 24 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.787C>T | p.Arg263* | 17 mo | 1 yrs | 14 yrs | 22 yrs | IV | II | ↓ | - |
| 42 | LBSL15 | 36 | f | 33 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.788G>A | p.Arg263Gln | 14 mo | 3 yrs | 8 yrs | 20 yrs | IV | III | ↓ | - |
| 43 | LBSL14 | 36 | f | 29 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.788G>A | p.Arg263Gln | 15 mo | 5 yrs | 28 yrs | - | III | II | nl | - |
| 44 | LBSL207 | 37 | m | 17 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.796C>T | p.Arg266* | 13 mo | 3 yrs | - | - | I | I | nl | - |
| 45 | LBSL119 | 38 | m | 22 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs5 | c.818_821del | p.Arg274Serfs*8 ^g | 15 mo | 14 yrs | - | - | II | II | nl | - |
| 46 | LBSL247 | 40 | m | 13 yrs | c.228-21_-20delTTinsC | p.Arg76Serfs*5 | c.1619C>G | p.Ser540* | - | 4 yrs | - | - | - | - | nl | - |
| 47 | LBSL223 | 39 | m | 4 yrs | c.228-21_-20delinsCC | p.Arg76Serfs*5 | c.1345-17_-5del | p.Cys449_Lys521del | 12 mo | 1.8 yrs | 22 mo | - | III | II | nl | - |
| 48 | LBSL70 | 41 | f | 23 yrs | c.228-20_-16delinsC- CCCA (c.228-16C>A) | p.Arg76Serfs*5 | c.295-2A>G | p.Ala100_Pro132del | 15 mo | 14 yrs | - | - | III | I | ↓ | - |
| 49 | LBSL266 | 42 | m | 13 yrs | c.228-20_-16delinsC- CCCA | p.Arg76Serfs*5 | c.455G>T | p.Cys152Phe | never | 0.9 yrs | never walked | never walked | V | IV | ↑↓ | - |

| Patient | Family | Gender | Age ^a | Mutation 1 ^b | Amino acid change | Mutation 2 ^b | Amino acid change | Unsup-ported walking | First neu- rological signs | Loss of un- supported walking | Loss of sup- ported walking | GMFCS ^c | MACS ^d | Learning ability ^e | Death | |
|---------|---------|--------|------------------|-------------------------|---|-------------------------|-------------------|------------------------------|----------------------------|-------------------------------|-----------------------------|--------------------|-------------------|-------------------------------|-------|---------|
| 50 | LBSL191 | 43 | m | 17 yrs | c.228-20_-16delinsC- CCCCA | p.Arg76Serfs*5 | c.716T>C | p.Leu239Pro | 18 mo | 2 yrs | 13 yrs | - | II | nl | - | |
| 51 | LBSL229 | 44 | f | 43 yrs | c.228-20_-16delinsC- CCCCA | p.Arg76Serfs*5 | c.1876C>G | p.Leu626Val | 14 mo | 40 yrs | - | - | II | I | nl | - |
| 52 | LBSL275 | 45 | f | † | c.228-20_-16delinsC- CCCCG | p.Arg76Serfs*5 | c.822_825del | p.Arg274Serfs*8 ^g | never | 0.4 yrs | never walked | never walked | V | V | n/c | 1.7 yrs |
| 53 | LBSL33 | 46 | f | 34 yrs | c.228-20_-15delinsC- CCCCA (c.228-15C>A) | p.Arg76Serfs*5 | c.133A>G | p.Ser45Gly | 24 mo | 6 yrs | 16 yrs | 25 yrs | IV | III | nl | - |
| 54 | LBSL259 | 47 | f | 8 yrs | c.228-20_-15delinsC- CCCCA | p.Arg76Serfs*5 | c.749T>C | p.Leu250Pro | 25 mo | 1 yrs | 5 yrs | 5.5 yrs | V | V | ↓ | - |
| 55 | LBSL64 | 48 | m | 15 yrs | c.228-20_-15delinsC- CCCCG (c.228-15C>G) | p.Arg76Serfs*5 | c.1886A>G | p.Tyr629Cys | nl | 4 yrs | - | - | I | I | nl | - |
| 56 | LBSL263 | 49 | f | † | c.228-20_-12delinsC- CCCCCCCCG | p.Arg76Serfs*5 | c.492+2T>C | p.Met134_Lys165del | never | 0.7 yrs | never walked | never walked | V | V | n/c | 1.8 yrs |
| 57 | LBSL210 | 50 | f | 34 yrs | c.228-20_-12delinsC- CCCCCCCCG | p.Arg76Serfs*5 | c.760G>A | p.Gly254Ser | 22 mo | 2 yrs | 30 yrs | - | III | II | nl | - |
| 58 | LBSL202 | 51 | m | 19 yrs | c.228-20_-12delinsC- CCCCCCCCCA | p.Arg76Serfs*5 | c.1069C>T | p.Gln357* | 13 mo | 10 yrs | - | - | II | II | nl | - |
| 59 | LBSL11 | 52 | f | 45 yrs | c.228-20_-11delinsC- CCCCCCCCG (c.228-11C>G) | p.Arg76Serfs*5 | c.536G>A | p.Arg179His | 15 mo | 5 yrs | 12 yrs | 20 yrs | V | IV | nl | - |
| 60 | LBSL203 | 53 | f | 20 yrs | c.228-20_-11delinsC- CCCCCCCCG | p.Arg76Serfs*5 | c.617_663del | p.Phe207Cysfs*25 | 18 mo | 1 yrs | - | - | II | II | nl | - |
| 61 | LBSL216 | 54 | m | 4 yrs | c.228-20_-10delinsC- CCCCCCCCCA | p.Arg76Serfs*5 | c.455G>T | p.Cys152Phe | 12 mo | 3 yrs | - | - | I | I | nl | - |
| 62 | LBSL111 | 55 | f | 16 yrs | c.228-20_-10delinsC- CCCCCCCCCA (c.228-10C>A) | p.Arg76Serfs*5 | c.492+2T>C | p.Met134_Lys165del | 14 mo | 13 yrs | - | - | I | II | nl | - |
| 63 | LBSL208 | 56 | f | 7 yrs | c.228-20_-10delinsC- CCCCCCCCCA | p.Arg76Serfs*5 | c.1726C>T | p.Pro576Ser | 15 mo | 4 yrs | - | - | I | II | nl | - |
| 64 | LBSL161 | 57 | m | 23 yrs | c.406A>T | p.Thr136Ser | c.172C>G | p.Arg58Gly | 22 mo | 3 yrs | - | - | II | II | ↓↓ | - |
| 65 | LBSL160 | 57 | m | 15 yrs | c.406A>T | p.Thr136Ser | c.172C>G | p.Arg58Gly | 24 mo | 2 yrs | - | - | II | II | ↓ | - |
| 66 | LBSL50 | 58 | f | 36 yrs | c.1837C>T | p.Leu613Phe | c.1877T>A | p.Leu626Gln | 18 mo | 12 yrs | 28 yrs | - | III | III | ↓ | - |

M, male; f, female; nl, normal; mo, months; yrs, years; n/c, not evaluable

M, male; f, female; nl, normal; mo, months; yrs, years; n/c, not evaluable

^a Age at latest clinical evaluation; ^b Nomenclature according to <http://www.hgvs.org/mutnomen/>. Mutations that were published before with a different annotation are shown between brackets;

^c Gross Motor Function Classification System⁸⁴; ^d Manual Ability Classification System⁸³; ^e Learning ability; nl, normal intelligence; ↓, mild learning problems, special education required;

↓↓, serious intellectual disability; ^f In this patient a third pathogenic mutation was found: c.1273G>T, p. Glu425*,^g p. Arg274Serfs*9 in current HGVS nomenclature.

Supplementary Table 4 Top 5 hits of compound screen

| Compound | YFP/mCherry ratio* |
|-------------------|--------------------|
| Gentian violet | 0,53 |
| Cantharidin | 0,59 |
| Pyrrithione zinc | 0,61 |
| Celastrol | 0,63 |
| Alanyl-dl-leucine | 0,71 |

*Control values observed in DMSO treated cells were set at a ratio of 1.